



PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re the Application of

BARNETT et al.

For: **POLYNUCLEOTIDES ENCODING
ANTIGENIC HIV TYPE C
POLYPEPTIDE, POLYPEPTIDES AND
USES THEREOF**

Serial No.: 09/610,313

Filed: July 5, 2000

Atty. Docket No.: PP01631.101 (2302-1631.20)

Examiner: B. Whiteman

Group Art Unit: 1633

Confirmation No.: 4221

**DECLARATION PURSUANT
TO 37 C.F.R. § 1.132**

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313

Sir:

I, John J. Donnelly, hereby declare as follows:

1. I received my Bachelors of Science Degree in Biology from the University of Pennsylvania in 1975 and a Doctorate of Philosophy Degree in Immunology in 1979 from the University of Pennsylvania. I also have a Masters of Sciences Degree in Strategic Studies from the U.S. Army War College.

2. I am currently Senior Director, Vaccine Research and Development in the Department of immunology & Infectious Diseases at Chiron Corporation and have been at Chiron since 1998. Before joining Chiron, I was Associate Director, Immunology Dept. of Virus & Cell Biology at Merck. Additional details regarding my background and qualifications can be found in the accompanying copy of my *Curriculum Vitae* (Exhibit A).

3. I have reviewed pending Patent Application Serial No. 09/610,313 for "POLYNUCLEOTIDES ENCODING ANTIGENIC HIV TYPE C POLYPEPTIDE,

POLYPEPTIDES AND USES THEREOF" by Barnett, et al., (hereinafter "the specification") and the currently pending claims. I have also reviewed the Office Action dated March 4, 2003. Therefore, I am familiar with the issues raised by the Examiner in the Office Action.

4. I understand that the pending claims are directed to expression cassettes comprising nucleotide sequences that encode immunogenic HIV Pol polypeptides. Further, the Pol-encoding nucleotide sequence must exhibit at least 90% identity to the sequences of SEQ ID NOs:30-32. It is further my understanding that the claims are also directed to cells comprising these polynucleotides and to methods of generating an immune response in a subject using the claimed polynucleotide sequences.

5. It is my opinion that, as a technical matter, a skilled worker could have readily made and used the compositions and methods of the pending claims in light of the specification, together with the common general knowledge, tools and methods available in July 2000. I base this opinion on the data and facts set forth below; however, I call attention to the fact that it was considered routine experimentation at the time of filing to determine a sequence having (i) at least 90% sequence identity to SEQ ID NO:30-32 and (ii) encoding an immunogenic Pol polypeptide; to express such polynucleotides in stem cells or their progenitors; to deliver in a variety of ways such polynucleotides to generate an immune response in a subject. In addition, in drawing my conclusions, I have considered the nature of the claims, the quantity of experimentation required to practice the subject matter of the claims, the existence of working examples, the direction present in the specification, the state of the field at the time the application was filed and the level of skill in the art.

6. At the outset, I note that the term "skilled worker" with a routine level of skill in the field of molecular biology, immunology and nucleic acid delivery in July 2000 had a Ph.D. degree and two or more years of post-doctoral training. In view of my training and experience, I am currently, and was in July of 2000, such a skilled worker.

7. In July 2000, the quantity of experimentation required to identify sequences exhibiting 90% identity to any given sequence, for example SEQ ID NOs:30-32, was quite low. For example, BLAST software programs were commonly known and readily available on the

Internet at this time. This set of programs allows for an easy alignment and determination of percent identity as between any sequences. The skilled worker could have easily used the BLAST or any number of other similar programs to determine the percent identity between sequences (in this case between any given sequence and those presented SEQ ID NOs:30-32). The specification also provides extensive guidance in this regard, for example, on page 19, line 19 to page 21, line 15. Working examples are also provided -- indeed, the specification provides four sequences falling within the scope of the claims (SEQ ID NOs:30-32 and 37). Furthermore, the skilled worker could have readily generated any sequence falling within the scope of the claims using routine methods, for example by utilizing PCR to generate sequences, by introducing point mutations and the like. Thus, it is my opinion that it would have required only routine experimentation to determine sequences falling within the 90% identity, as claimed.

8. In addition, the specification provides significant direction for evaluating whether sequences having 90% identity to SEQ ID NO:30-32 encode an immunogenic Pol polypeptide. Those of us working in the field of gene delivery and immunology are well versed in the various tests for determining immunogenicity, which include computer analysis of sequences, comparison to known immunogenic sequences as well as functional tests (*e.g.*, ELISAs, CTL assays and others described in the Examples of the specification). Pol antigens or antibodies recognizing Pol antigens had long been used to test for Pol-stimulated immune responses (*e.g.*, *n* immunoassays). ELISPOT assays for testing cellular immunity were also well known at the time of filing.

9. Furthermore, the state of the art in July 2000 was quite sophisticated with regard to determining both sequence identity and evaluating immunogenicity. I have described above some of the tools, programs and methods available in the field of recombinant nucleic acid technology in July 2000 and many other examples of homologous nucleic acid molecules that encode immunogenic proteins were also available. Therefore, it is my opinion that, following the guidance of the specification, a scientist could have readily made and used polynucleotide sequences that exhibit at least 90% sequence identity to SEQ ID NO:30-32 and which encode an immunogenic HIV Pol polypeptide.

10. Preparing polynucleotides encoding immunogenic Pol polypeptides in July 2000 was a predictable art. There is no doubt that a skilled worker would have been able to make and use sequences exhibiting 90% identity to SEQ ID NO:30-32 and encoding an immunogenic polypeptide. Even if a rare construct were inoperable for some reason (*e.g.*, it wasn't immunogenic), the skilled worker would have readily modified the construct according to the alternatives available at the time and described in the specification. In other words, to the skilled worker, an inoperable construct would itself be a useful starting material for other operable constructs. Essentially all molecules that fall within the claims would be useful for making or using defining technical features of the claims, *i.e.*, nucleotide sequences having 90% sequence identity to SEQ ID NO:30-32 and which encoded an immunogenic HIV Pol polypeptide.

11. Similarly, the specification as filed clearly provides ample guidance on how to generate an immune response (humoral and/or cellular) in a subject by administering the claimed sequences. (See, page 7, lines 12 to 23; and Examples 4 and 7). Indeed, in July 2000, it was predictable and routine to evaluate whether an immune response was generated against a polypeptide antigen encoded by an administered polynucleotide, for example using the techniques and tools described above in paragraph 8. Furthermore, the skilled worker would know that generating an immune response does not necessarily mean that the subject will be vaccinated – *i.e.*, protected against HIV infection or derive some therapeutic benefit. The skilled worker would also have known that immune responses are useful for numerous scientific purposes, such as laboratory assays, preparing reagents for virologic and immunologic studies, analyzing immune responses, and preparation of diagnostic kits. Therefore, a skilled worker would have known that the claimed sequences could be used for additional scientific purposes other than seeking protective immunity or a therapeutic benefit. In view of the guidance in the specification, the predictability and state of the art, and high level of the skilled worker, it is plain that it would have been routine to administer a polynucleotide and evaluate whether or not an immune response to the encoded polypeptide was generated in the subject.

12. Experiments conducted in our laboratories demonstrate that expression cassettes that include modified HIV Pol-encoding sequences induce potent Pol-specific immune

responses. These experiments are summarized in zur Megede et al. (2002) *J. Virol.* 77(11):6197-6207, attached hereto as Exhibit B. As shown in Exhibit B, we generated modified Pol-encoding sequences from subtype B isolates of HIV using the protocols described in the specification. (See, Example 1). Also using techniques set forth in the specification, we inserted these modified Pol-encoding sequences into an expression cassette such that they are operably linked to a promoter. These expression cassettes were administered to living animals and immunogenicity evaluated, using the protocols set forth in the specification. Our results establish that "all of the sequence-modified pol and gagpol plasmids expressed high levels of Pol-specific antigens in a Rev-independent fashion and we were able to induce potent Pol-specific T- and B-cell responses..." (Abstract of Exhibit B). In light of our results, I conclude that modified HIV Pol-encoding sequences can be inserted into expression cassettes such that they are operably linked to a promoter and that these expression cassettes are immunogenic. I also conclude that a variety of sequences exhibiting 90% homology to each other are equally effective. Furthermore, because Pol-encoding sequences can be obtained from any HIV isolate and modified as described in the specification, the results we presented in Exhibit B with regard to subtype B sequences are equally applicable to modified polynucleotides obtained from subtype C isolates, as claimed.

13. It would have also been routine to express the claimed Pol-encoding polynucleotides in stem cells or lymphoid progenitor cells. The guidance in the specification in this regard is extensive. In addition, the level of skill in this field was very high at the time of filing, the state of the art sophisticated and the experimentation needed to get expression in lymphokine cells (such as stem cells and lymphoid progenitor cells) was routine using standard vectors (e.g., plasmids such pBR322 and pBLUESCRIPT that include promoters and other control elements). Even a reference cited in the Office Action makes it clear that heterologous HIV polypeptide-encoding sequences can readily be introduced into and expressed in stem cells:

Other areas where gene transfer into hematopoietic cells is being investigated include human immunodeficiency virus (HIV) infection ... the importance of these studies cannot be over emphasized as they provide 'proof-in-principle' that gene-marked cells can survive and be expressed for extended periods of time once

re-introduced into the host. (Prince, *Pathology* 30:335-347 at page 340, left column, emphasis added).

Therefore, the specification teaches a skilled worker how to express the claimed Pol-encoding sequences in stem cells or progenitors of lymphoid cells.

14. Furthermore, I believe that, following the teachings of the specification and guidance of the art, a skilled worker could have readily administered the claimed nucleic acids specification by a variety of modes including intramuscular, intradermal, mucosal and the like. The quantity of experimentation required to use alternatives to intramuscular delivery routes was quite low in July 2000. A skilled worker could have easily administered polynucleotides by a variety of routine methods known at the time of filing. For example, administration of polynucleotides encoding HIV antigens via intradermal and mucosal modes is described in Shiver et al. 1997 *Vaccine* 15:884-887 (Exhibit C) and Durrani et al. 1998 *J. Immunol. Methods* 220:93-103 (Exhibit D). These references are clearly representative of the high level of skill in the art and the fact that non-intramuscular modes of administration were considered predictable in July 2000 -- many of the examples gene delivery modes were also known. Furthermore, at the time of filing, it was known in the art that administration of polynucleotide vaccines by diverse routes such as intradermal, transdermal, intranasal, oral and the like did not require special modifications to the coding sequence of the polynucleotide plasmid construct itself. The specification provides significant direction in these regards as well, for example on page 61 of the specification. Therefore, a skilled worker would have found the claimed expression cassette and sequences at least 90% identical to it to be useful for generating an immune response using diverse routes and methods. Thus, to the skilled worker, administering the claimed polynucleotides by any number of delivery routes would have been routine and required only minor experimentation.

15. It is also my opinion that the specification as filed clearly conveyed to a typical scientist that the inventors had in their possession the invention set forth in the claims (see paragraph 4 above). By "in their possession," I mean that the inventors contemplated the polynucleotides, cells and methods as set forth in the claims and that they had, using the

specification and information available to a typical scientist, a practical way of making such molecules and practicing such methods. Thus, I believe that a typical scientist would have understood the specification clearly described all of the various aspects of the claims. I base this belief on the facts set forth herein.

16. First, the specification unambiguously and clearly describes at the time the specification was filed, it was widely known how to determine sequence identity to any length polynucleotides. Such methods are described in detail in the specification, for example, on pages 19-21 of the specification. (see, also, paragraph 7 above). Therefore, it is my opinion that the specification describes any sequence exhibiting 90% sequence identity to SEQ ID NOs:30-32.

17. Second, at the time the specification was filed, it would have been clear to a typical scientist that the inventors' specification fully described and contemplated that the claimed polynucleotides encoded immunogenic Pol polypeptides. Methods of testing Pol immunogenicity were well-known at the time of filing and are demonstrated, for example, in Exhibit B. Indeed, our experiments, presented in Exhibit B, indicate that Pol-specific immune responses are generated to the claimed sequences. In sum, based on the disclosure of the specification and the level of knowledge of a typical scientist regarding sequence identity, and testing for immunogenicity, I believe that the specification as filed clearly conveys that the applicants had invented the expression cassettes as set forth in the claims.

18. In view of the foregoing facts regarding the routine nature of experimentation required to make and use the claimed constructs, the extensive direction provided by the specification, the straightforward nature of the invention, the presence of working examples, the high level of the skilled worker, the sophistication of the art, and the predictability (*e.g.*, of determining sequences identity and immunogenicity) of the art, it is my unequivocal opinion that the specification enabled, in July 2000, a skilled worker to make and use the subject matter of the claims. Similarly, in view of the detailed description in the specification and state of the field at the time of filing, it is my opinion that the specification more than adequately conveys that the inventors had possession of the claimed polynucleotides, expression cassettes, vectors, cells and methods of generating immune responses at the time of filing in July 2000.

19. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

29 AUG 03
Date

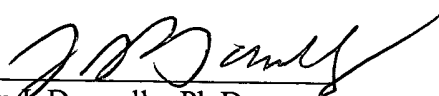

John F. Donnelly, Ph.D.

EXHIBIT A

CURRICULUM VITAE

I. PERSONAL

A. Name: John J. Donnelly

B. Address: 46 Fieldbrook Pl
Moraga, CA 94556

C. Home Telephone: (925)376-5602
Office Telephone: (510)923-8371
FAX: (978)359-8021
E-mail: john_donnelly@chiron.com

II. EDUCATION

<u>School</u>	<u>Date</u>	<u>Field</u>	<u>Degree</u>
University of Pennsylvania	1971-1975	Biology	B.A.
University of Pennsylvania	1975-1979	Immunology	Ph.D.
US Army War College	2000-2002	Strategic studies	M.S.

III. TRAINING

<u>Source</u>	<u>Date</u>	<u>Type</u>
Department of Ophthalmology John Hopkins University School of Medicine Baltimore, Maryland	1982	Postdoctoral Research Fellow (Preceptor: R.A. Prendergast, M.D.)
Department of Clinical Veterinary Medicine University of Cambridge Cambridge, England	1980-81	Postdoctoral Research Fellow (Preceptor: Prof. E.J.L. Soulsby, D.V.S.M., M.R.C.V.S., Ph.D.)

IV. SOCIETY MEMBERSHIPS

American Association of Blood Banks
American Association of Immunologists
Association for Research in Vision and Ophthalmology
British Society for Immunology
New York Academy of Sciences
Royal Society for Tropical Medicine and Hygiene

V. ACADEMIC AND PROFESSIONAL HONORS

2000 President's Leadership Award, Chiron Corp.
1976-79 NIH Predoctoral Traineeship
1977 Fight for Sight Student Fellow
1980-81 Fight for Sight Postdoctoral Research Fellow

- 1982 NIH Individual Postdoctoral Fellowship
- 1983 Robert E. Shoemaker Research Award, Pennsylvania Academy of Ophthalmology and Otolaryngology
- 2000 President's Leadership Award, Chiron Research and Development

VI. ACADEMIC EXPERIENCE

A. Within the last five years

1988 - 1998 Adjunct Assistant Professor
Department of Ophthalmology
University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania

B. Prior to the last 5 years

1983-88 Assistant Professor, Department of Ophthalmology
University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania

1986-88 Graduate Group in Immunology
University of Pennsylvania
Philadelphia, Pennsylvania

1983-88 Graduate Group in Parasitology
University of Pennsylvania
Philadelphia, Pennsylvania

VII. EMPLOYMENT HISTORY

Position Title: Senior Director, Vaccine Research and Development
Department of immunology & Infectious Diseases
Chiron Research and Development
Chiron Corporation

Duration: July 2000-present

Brief Description of Significant Responsibilities:

Manage more than 20 Principal and Associate Scientists in research on HIV Vaccines and vaccine adjuvants and delivery. Direct Chiron HIV vaccine research and development program. Lead team responsible for externally financing HIV Vaccine R&D project; raised over \$42 million of outside funds, mostly from NIH, since 1999. Direct clinical serology laboratory supporting Phase I-II studies of N. meningitidis group B vaccine. Provide research support for clinical studies of therapeutic hepatitis B vaccine and Interleukin-2 therapy of HIV. Direct basic research on serologic markers for immunity to Neisseria meningitidis group B. Direct basic research in cancer immunotherapy. Responsibilities include direction of basic research, direction of preclinical studies, budgeting, contribution to regulatory documentation, and presentations to outside regulatory groups such as the FDA Vaccines and Related Biologicals Advisory Committee. Chair of Institutional Animal Care and Use Committee for Chiron Corp, responsible for Emeryville and Seattle sites.

Position Title: Acting Vice President, Vaccine Research and Development
Chiron Research and Development
Chiron Corporation

Duration: February - July 2000

From departure of Vice President until new Vice President brought in from Chiron Siena, managed more than 35 Principal and Associate Scientists in research on HIV Vaccines, HCV Vaccines, DNA Vaccines, Vaccine Adjuvants and Delivery, and cell culture and recombinant protein production. Restructured Vaccines Research Department to achieve fiscal balance. Retained key personnel while reducing workforce by 15%. Managed internal and external HIV vaccine research and development activities. Directed clinical serology laboratory supporting Phase III studies of meningitis C conjugate vaccine (Menjugate®). Provided research support for clinical studies of therapeutic hepatitis B vaccine and Interleukin-2 therapy of HIV. Directed basic research on serologic markers for immunity to Neisseria meningitidis group B. Responsibilities included direction of basic research, direction of preclinical studies, budgeting, contribution to regulatory documentation, and presentations to outside regulatory groups such as the FDA Vaccines and Related Biologicals Advisory Committee. Chaired Institutional Animal Care and Use Committee for Chiron Corp.

Position Title: Director, Vaccine Adjuvants Research
Chiron Technologies
Chiron Corporation

Duration: 1998-presentBrief Description of Significant Responsibilities:

Manage more than 30 Principal and Associate Scientists in research on vaccine adjuvants, induction of cytotoxic T cells, DNA vaccines for HIV and HCV, cancer immunotherapy, gene therapy with MuLV-based viral vectors, and bacterial vaccines. Direct internal and external adjuvant research programs. Responsibilities include direction of basic research, direction of preclinical studies, budgeting, contribution to regulatory documentation, and presentations to outside regulatory groups such as the FDA Vaccines and Related Biologicals Advisory Committee. Beginning in September 1999, chaired Institutional Animal Care and Use Committee for Chiron Corp.

Position Title: Associate Director, Immunology
Dept. of Virus & Cell Biology
Merck Research Laboratories

Duration: 1994-98Brief description of significant responsibilities:

Manage more than 10 Principal and Associate Scientists in basic research on DNA vaccines for influenza, HCV, and HPV, recombinant protein vaccines for Hepatitis B, vaccine adjuvants, and preclinical and clinical studies for Haemophilus influenzae type B and Streptococcus pneumoniae polysaccharide-protein conjugate vaccines. Studied cytotoxic T cell responses in nonhuman primates and cytokine responses in human subjects to experimental influenza DNA vaccines. Prepare regulatory documentation including preclinical sections of PLA's and Part III (Pharmaco-toxicological Documentation) of MAA's for bacterial vaccines and combination vaccines (Liquid PedvaxHIB®, COMVAX®, New Process Pneumovax 23®). Chaired Institutional Animal Care and Use Committee for West Point site.

Position Title: Research Fellow
Dept. of Virus & Cell Biology
Merck Research Laboratories

Duration: 1988-94

Brief Description of Significant Responsibilities:

Supervise up to 8 Principal and Associate Scientists in research on mechanisms of induction of cytotoxic T lymphocytes, including immunization with DNA, evaluation of adjuvants for clinical use in vaccines, development of analytical/serological assays for support of clinical vaccine programs (HPV, HIV, Influenza), preclinical development of bacterial vaccines, and preclinical development of influenza DNA vaccines. Direct research/licensing program in vaccine adjuvants and delivery systems.

- 1980-81 Postdoctoral Research Fellow, Department of Clinical Veterinary Medicine
University of Cambridge, Cambridge, England
- 1982 Postdoctoral Research Fellow, Department of Ophthalmology
Johns Hopkins University School of Medicine
Baltimore, Maryland
- 1983-88 Assistant Professor, Department of Ophthalmology
University of Pennsylvania School of Medicine
Philadelphia, Pennsylvania

IX. OTHER SKILLS, QUALITIES OR ACCOMPLISHMENTS

A. Membership on Peer Review Panels:

- | | |
|---|-----------|
| USAID Biotechnology/Immunology Panel | 1988-1991 |
| NIH/NIAID Review Committee for RFA NIH-NIAID-94-11,
Basic Biology of Immune Responses for Vaccine Research | 1994 |
| NIH/NIAID Visual Sciences A Study Section, Ad hoc member | 1997 |
| NIH/NIDR Special Emphasis Panel on Oral Carcinoma | 1997 |
| USAID Schistosomiasis Vaccine Development Program Advisory
Group, 4 year term beginning | 1997 |
| NIH/NIAID Vaccines Study Section, 3 year term beginning | 1998 |

B. Editorial Boards:

- | | |
|--|-------|
| Contributing Editor: | |
| Autoimmunity | 1988- |
| Current Eye Research | 1987- |
| Cellular Immunology | 1986- |
| Investigative Ophthalmology and Visual Science | 1981- |
| Journal of Immunology | 1994- |

C. Meetings Organized

- | | |
|--|------|
| IBC First Annual Conference on Genetic Vaccines | 1995 |
| IBC Second Annual Conference on Genetic Vaccines | 1996 |
| IBC 4th Annual International Conference on
Mucosal Immunization | 1996 |
| IBC Third Annual Conference on Genetic Vaccines | 1997 |
| IBC Fourth Annual Conference on Genetic Vaccines | 1998 |
| 2 nd Annual US Biotechnology Symposium | 1999 |

D. Military Service:

Colonel, Medical Service Corps, U.S. Army Reserve

Blood Program Officer, Third United States Army (Forward), King Khalid
Military City, Saudi Arabia, 12/22/90-4/1/91

X. PUBLICATIONS AND PATENTS

Donnelly, J.J., Rockey, J.H. and Soulsby, E.J.L.: Intraocular IgE antibody induced in guinea pigs with *Ascaris suum* larvae. Invest. Ophthalmol. Vis. Sci. 16: 976-981, 1977.

Rockey, J.H., Donnelly, J.J., Stromberg, B.E. and Soulsby, E.J.L.: Immunopathology of *Toxocara canis* and *Ascaris suum* infections of the eye: The role of the eosinophil. Invest. Ophthalmol. Vis. Sci. 18: 1172-1184, 1979.

Soulsby, E.J.L., Stromberg, B.E., Donnelly, J.J. and Rockey, J.H.: Intraocular immunoglobulin E induced by intravitreal infection with *Ascaris* and *Toxocara* spp. larvae. Ophthal. Res. 12: 45-53, 1980.

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Donnelly, J.J., Rockey, J.H., Bianco, A.E., and Soulsby, E.J.L.: Aqueous humor and serum IgE antibody in experimental ocular *Onchocerca* infection of guinea pigs. Ophthal. Res. 15: 61-67, 1983.

Rockey, J.H., John, T., Donnelly, J.J., McKenzie, D.F., Stromberg, B.E., and Soulsby, E.J.L.: In vitro interactions of eosinophils from ascarid-infected eyes with *A. suum* and *T. canis* larvae. Invest. Ophthalmol. Vis. Sci. 24: 1346-1357, 1983.

John, T., Donnelly, J.J. and Rockey, J.H.: Experimental ocular *Toxocara canis* and *Ascaris suum* infection: In vivo and in vitro study. Trans. Pa. Acad. Ophthalmol. Otolaryngol. 36: 131-137, 1983.

Attenburrow, D.P., Donnelly, J.J. and Soulsby, E.J.L.: Periodic ophthalmia (recurrent uveitis) of horses: An evaluation of the etiological role of microfilariae and the clinical management of the condition. Equine Vet. Journal 15: 48-56, 1983.

Donnelly, J.J., Rockey, J.H., Bianco, A.E. and Soulsby, E.J.L.: Ocular immunopathologic findings of experimental onchocerciasis. Arch. Ophthalmol. 102: 628-634, 1984.

Donnelly, J.J. and Prendergast, R.A.: Local production of Ia-inducing activity in experimental immunogenic uveitis. Cellular Immunology 86: 557-561, 1984.

Khatami, M., Donnelly, J.J., John, T. and Rockey, J.H.: Vernal conjunctivitis. Model studies on guinea pigs immunized topically with fluoresceinyl ovalbumin. Arch. Ophthalmol. 102: 1683-1688, 1984.

Lok, J.B., Pollack, R.J., Cupp, E.W., Bernardo, M.J., Donnelly, J.J., and Albiez, E.J.: Development of third-stage larvae of *Onchocerca volvulus* and *O. lienalis* in vitro. Tropenmedizin und Parasitologie 35: 209-212, 1984.

Donnelly, J.J., Vogel, S.N. and Prendergast, R.A.: Down-regulation of Ia expression on macrophages by Sea Star Factor. Cellular Immunology 90: 408-415, 1985.

Rockey, J.H., Donnelly, J.J., John, T., Khatami, M., Schwartzman, R.M., Stromberg, B.E., Bianco, A.E. and Soulsby, E.J.L.: IgE antibodies in ocular immunopathology. Third International Symposium on the Immunology and Immunopathology of the Eye. Masson, New York, pp.199-202, 1985.

Khatami, M., Donnelly, J.J. and Rockey, J.H.: Induction and down-regulation of conjunctival Type-I hypersensitivity reactions in guinea pigs sensitized topically with fluoresceinyl ovalbumin. *Ophthalmic Research* 17: 139-147, 1985.

Donnelly, J.J., Li, W., Rockey, J.H. and Prendergast, R.A.: Induction of class II (Ia) alloantigen expression on corneal endothelium *in vivo* and *in vitro*. *Invest. Ophthalmol. Vis. Sci.* 26: 575-580, 1985.

Donnelly, J.J., Rockey, J.H., Taylor, H.R. and Soulsby, E.J.L.: Onchocerciasis: Experimental models of ocular disease. *Reviews of Infectious Diseases* 7: 820-825, 1985.

Donnelly, J.J., Taylor, H.R., Young, E.M., Khatami, M., Lok, J.B. and Rockey, J.H.: Experimental ocular onchocerciasis in cynomolgus monkeys. *Invest. Ophthalmol. Vis. Sci.* 27: 492-499, 1986.

Sakla, A.A., Donnelly, J.J., Lok, J.B., Khatami, M. and Rockey, J.H.: Punctate keratitis induced by subconjunctivally injected microfilariae of *Onchocerca lienalis*. *Arch. Ophthalmol.* 104: 894-898, 1986.

James, E.R., Smith, B. and Donnelly, J.J.: Invasion of the mouse eye by *Onchocerca* microfilarie. *Trop. Med. and Parasitol.* 37: 359-360, 1986.

Donnelly, J.J., Sakla, A.A., Hill, D.E., Lok, J.B., Khatami, M. and Rockey, J.H.: Effects of diethylcarbamazine citrate and anti-inflammatory drugs on experimental onchocercal punctate keratitis. *Ophthalmic Research*. 19: 129-136, 1987.

Lok, J.B., Pollack, R.J. and Donnelly, J.J.: Studies of the growth-regulating effects of Ivermectin on larval *O. lienalis* *in vitro*. *J. Parasitol.* 73: 80-84, 1987.

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Donnelly, J.J., Xi, M.-S., Haldar, J.P., Hill, D.E., Lok, J.B., Khatami, M. and Rockey, J.H.: Autoantibody induced by experimental *Onchocerca* infection: Effects of different routes of administration of microfilariae and of treatment with diethylcarbamazine citrate and Ivermectin. *Invest. Ophthalmol. Vis. Sci.* 29: 827-831, 1988.

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EXHIBIT B

Expression and Immunogenicity of Sequence-Modified Human Immunodeficiency Virus Type 1 Subtype B *pol* and *gagpol* DNA Vaccines

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Control of the worldwide AIDS pandemic may require not only preventive but also therapeutic immunization strategies. To meet this challenge, the next generation of human immunodeficiency virus type 1 (HIV-1) vaccines must stimulate broad and durable cellular immune responses to multiple HIV antigens. Results of both natural history studies and virus challenge studies with macaques indicate that responses to both Gag and Pol antigens are important for the control of viremia. Previously, we reported increased Rev-independent expression and improved immunogenicity of DNA vaccines encoding sequence-modified Gag derived from the HIV-1_{SF2} strain (J. zur Megede, M. C. Chen, B. Doe, M. Schaefer, C. E. Greer, M. Selby, G. R. Otten, and S. W. Barnett, *J. Virol.* 74: 2628–2635, 2000). Here we describe results of expression and immunogenicity studies conducted with novel sequence-modified HIV-1_{SF2} GagPol and Pol vaccine antigens. These Pol antigens contain deletions in the integrase coding region and were mutated in the reverse transcriptase (RT) coding region to remove potentially deleterious enzymatic activities. The resulting Pol sequences were used alone or in combination with sequence-modified Gag. In the latter, the natural translational frameshift between the Gag and Pol coding sequences was either retained or removed. Smaller, in-frame fusion gene cassettes expressing Gag plus RT or protease plus RT also were evaluated. Expression of Gag and Pol from GagPol fusion gene cassettes appeared to be reduced when the HIV protease was active. Therefore, additional constructs were evaluated in which mutations were introduced to attenuate or inactivate the protease activity. Nevertheless, when these constructs were delivered to mice as DNA vaccines, similar levels of CD8⁺ T-cell responses to Gag and Pol epitopes were observed regardless of the level of protease activity. Overall, the cellular immune responses against Gag induced in mice immunized with multigenic *gagpol* plasmids were similar to those observed in mice immunized with the plasmid encoding Gag alone. Furthermore, all of the sequence-modified *pol* and *gagpol* plasmids expressed high levels of Pol-specific antigens in a Rev-independent fashion and were able to induce potent Pol-specific T- and B-cell responses in mice. These results support the inclusion of a *gagpol* in-frame fusion gene in future HIV vaccine approaches.

The AIDS pandemic caused by human immunodeficiency virus type 1 (HIV-1) is believed to have cost 3.1 million lives in the year 2002 alone, with over 42 million people believed to be infected worldwide (<http://www.unaids.org/worldaidsday/2002/press/Epiupdate.html>). At present, 20 years after the discovery of HIV/AIDS, no effective HIV vaccine has been identified and few candidates have advanced beyond early-phase clinical trials (20). While effective drug therapy is available in developed parts of the world, it is financially out of reach for most of the world's population of infected individuals. It is thus widely believed that an efficacious prophylactic vaccine is critical for the control of the global spread of HIV/AIDS. Furthermore, therapeutic vaccine approaches in combination with drug therapy, which allow patients to be off drugs for extended periods of time, also hold great promise for those already infected (18, 45).

While the primary focus for first-generation HIV vaccines was the induction of neutralizing antibodies using HIV enve-

lope (Env)-based approaches, more recently, the focus has extended to the induction of CD8⁺ cytotoxic T-lymphocyte (CTL) responses against conserved internal viral antigens such as Gag and Pol (17). This shift was a result of studies of natural infections, long-term nonprogressors, and exposed uninfected individuals that have, in multiple studies, demonstrated an inverse correlation between the potency and breadth of CD4⁺ and CD8⁺ T-cell responses and HIV disease progression (7, 8, 14, 29, 31, 33, 44, 46). Moreover, vaccine approaches specifically designed to induce strong cellular immunity recently have shown promising results in nonhuman primate vaccine challenge models (2, 5, 49). In these studies, the induction of strong CD8⁺ T-cell responses against Gag in vaccinated macaques appeared to result in decreased viremia, morbidity, and mortality when animals were subsequently challenged with pathogenic simian/human immunodeficiency viruses. Nevertheless, this strategy of using gene-based vaccines alone to induce CD8⁺ T-cell responses does not appear to protect monkeys from infection and the challenge virus was able to eventually escape immune control, resulting in increased viremia and its sequelae (4, 24).

Interestingly, the use of prime-boost immunization strategies, including those that use Env antigens as the protein in

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several of these CTL-based vaccines, has repeatedly been shown to improve the degree of protection observed (23, 36, 37, 42, 43). Whether this is due to the priming of protective B- or T-cell responses has not been elucidated in these studies. In addition to the use of prime-boost strategies, the use of multiple genes in the vaccine to increase the number of potential T-cell epitopes has also improved the outcome after a virus challenge over that achieved with a single- or double-gene vaccine (2, 30). Therefore, the overall goal of our program has been to achieve the greatest breadth of cellular immunity directed to multiple HIV antigens in combination with broad neutralizing antibody responses, an approach that may be more successful at blocking infection than has been previously observed.

The goal of the present study was to evaluate the expression and immunogenicity of novel vaccine antigens based on portions of the HIV-1 Pol polyprotein administered alone or in combination with Gag. Pol is a conserved protein of HIV-1, and cross-clade CTL responses against Pol epitopes have been detected in both HIV-infected and exposed but uninfected individuals (6, 7, 47). The inclusion of the *pol* gene in the form of the *gagpol* precursor in earlier vaccine trials with humans and nonhuman primates was most likely suboptimal with regard to inefficient expression of the Pol antigen. The expression levels of the Pol protein generally are low during natural infection because of the frameshift required for translation of *pol* coding sequences. This mode of Pol expression results in an up to 95% reduction in Pol protein compared to Gag (27, 53). To increase Pol expression, the frameshift between *gag* and *pol* can be removed, resulting in equimolar or nearly equimolar expression of Gag and Pol whereas the secretion of virus-like particles (VLP) is impaired (28, 40). To evaluate the potential antigenic competition between Gag and Pol if they are encoded in one expression cassette, various expression cassettes were designed and tested with the antigens encoded on single or multigenic expression plasmids. Another consideration was the possible cytotoxic effect of the active viral protease and possible effects of the active protease on Gag and Pol antigen expression levels. Therefore, mutations known to attenuate or inactivate HIV-1 protease (32) were introduced. Additional safety features introduced into the *pol* expression cassette included the removal of integrase and the mutation of the reverse transcriptase (RT) to remove these potentially deleterious enzymatic activities.

Plasmid DNA vaccines encoding these sequence-modified *gag*, *pol*, and *gagpol* genes were evaluated for expression in vitro after transient transfection of 293 cells and subsequently in dose titration immunogenicity studies performed with mice. Overall, the cellular immune responses against Gag induced by the various multigenic Gag- and Pol-expressing plasmids were similar to those induced by the plasmid encoding Gag alone. All of the sequence-modified *pol* and *gagpol* plasmids expressed high levels of Pol-specific antigens in a Rev-independent fashion and were able to induce potent Pol-specific T-cell responses in mice. Moreover, removal of the frameshift between *gag* and *pol* resulted in increased expression of Pol and increased RT-specific immune responses, as expected. Lastly, while the activity of protease appeared to have an inhibitory effect on the expression of Gag and Pol antigens in vitro, the immunogenicities of constructs encoding active protease did

not appear to be reduced in mice. The CD8⁺ T-cell responses against Gag- and RT-specific epitopes, as measured by flow cytometric analysis of gamma interferon (IFN- γ)-producing cells, were comparable for all constructs regardless of the level of protease activity. These results support the inclusion of a sequence-modified in-frame *gagpol* fusion cassette in future HIV vaccine approaches.

MATERIALS AND METHODS

Plasmid DNA cassettes. A panel of expression cassettes based on the amino acid sequences of HIV-1_{SF2} subtype B Gag and Pol antigens was designed with sequence modifications as described previously (22, 56). All gene cassettes were cloned into eukaryotic expression vector pCMVkm2, which contained the cytomegalovirus immediate-early enhancer-promoter and the bGH terminator (Chiron Corporation, Emeryville, Calif.) (12). To further enhance the translation efficiency of all expression cassettes, an optimal "Kozak" consensus sequence (GCCACC) for initiation of translation was inserted (34). *gag*-only plasmid pCMVkm2.GagMod.SF2 (GenBank accession no. AF201927) and *gagprotease* cassettes GP1 and GP2 (pCMVkm2.GagProt.SF2; GenBank accession no. AF202464 and AF202465) have been described previously (56).

The entire integrase coding sequence in *pol* was deleted for safety reasons, and the catalytic triad and primer grip regions of the RT coding sequences were deleted to inactivate these enzymatic activities (39, 41). The construct gagFSpol was based on the GP2 cassette but was extended for *pol* up to the RNase H coding sequences. For the *gagpol* and *gag-complete-pol* (gagCpol) cassettes, the frameshift region was removed by insertion of an extra T nucleotide at the p1 "slippery sequence" (TTTTTA) in order to express the *gag* and *pol* genes in frame. The *pol* region included p1p6^{Pol} coding sequences up to RNase H for *gagpol*. To include p1p6^{Gag} and for optimal processing of Gag and Pol by the protease, the *p2p7p1p6* fragment was added to get gagCpol (see Fig. 1). The constructs gagRT and gagprotInaRT expressed fusion proteins of p55^{Gag} and either p66^{RT} or p10^{protease} plus p66^{RT}. Furthermore, gene cassettes for the expression of p66^{RT} alone and proteaseRT and p2p7^{Gag} plus p1p6^{Pol} (p2pol) were also included. When indicated, the protease in some constructs was either attenuated (Att) or inactivated (Ina) by the introduction of specific point mutations (32) with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.).

Testing for in vitro expression. Human kidney 293 cells (no. 45504; American Type Tissue Collection, Manassas, Va.) were plated 1 day prior to transfection at a density of 5×10^5 cells per 35-mm-diameter well (Corning, Acton, Mass.) and transfected with endotoxin-free purified plasmid DNA (Qiagen, Valencia, Calif.). For the transfections, 2 μ g of each plasmid DNA was mixed with Mirus TransIT-LTI Polyamine transfection reagent (PanVera, Madison, Wis.). The cells were incubated with 2 ml of 10% Iscove's modified Dulbecco's medium (Invitrogen, Carlsbad, Calif.) per well for 48 and 72 h, and the supernatants and lysates were then harvested for further analysis. Quantitation of p24^{Gag} protein in cell supernatants and lysates was performed with the Coulter p24 Antigen Capture enzyme-linked immunosorbent assay (ELISA; Coulter Corporation, Miami, Fla.). The Western blot assay for Gag and Pol expression analysis was done by using 4 to 12% Bis-Tris sodium dodecyl sulfate-polyacrylamide gels (Invitrogen) and then transfer onto 0.2- μ m-pore-size nitrocellulose (Invitrogen). Prestained full-range rainbow marker (Amersham, Piscataway, N.J.) and recombinant HIV-1 p24^{Gag}, p55^{Gag} (Chiron), and p66^{RT} (Protein Sciences, Meriden, Conn.) proteins were used as the size standard and positive controls, respectively. For detection of Gag proteins by immunostaining, membranes were incubated with HIV-1-positive human serum at a dilution of 1:400. For Pol proteins, an anti-p66^{RT} monoclonal antibody (MAb; 1:200; Fitzgerald, Concord, Mass.) and pooled mouse serum (1:400) against p66^{RT} (Chiron) were used. Secondary antibodies (1:20,000) were anti-human or anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Pierce, Rockford, Ill.). Detection was performed by using the enhanced chemiluminescence substrate (Amersham). The predicted molecular weights of the various expression cassettes tested were calculated from the predicted amino acid sequences by using MacVector software (Oxford Molecular Ltd.).

Immunization of mice. To evaluate the relative potencies of the immune responses induced by the different constructs, female CB6F1 or C3H/HeN mice, 6 to 8 weeks old, were immunized bilaterally in the tibialis anterior muscles with 100- μ l volumes of endotoxin-free plasmid DNA in isotonic saline (50 μ l per site). The DNA concentrations of the test plasmids were adjusted to provide equal molar quantities of Gag or Pol at a given DNA dose. Furthermore, all DNA of

TABLE 1. Overview of mouse studies

Expt no.	Fig. no.	Mouse strain	Vaccines ^a	Immunization (day[s])	rVV challenge (day)	Blood collection (days)
1	4	CB6F1	a, b, c	0	28	0, 28, 33
2	5A, 6B	CB6F1	a, d, f, g, h	0	28	0, 28, 33
3	5B	CB6F1	a, d, f, g, h	0, 28	None	0, 28, 42
4	6A	CB6F1	a, b, c	0, 28	None	0, 28, 42
5	7A	C3H/HeN	e, f, g, i, k	0	28	0, 28, 33
6	7C	C3H/HeN	e, f, g, i, k	0, 28	None	0, 28, 42
7	7B	C3H/HeN	d, f, g, h, l	0	28	0, 28, 33
8	7D	C3H/HeN	d, f, g, h, l	0, 28	None	0, 28, 42

^a Vaccines: a, gag; b, GP1; c, GP2; d, gagFSpol; e, gagRT; f, gagprotInaRT; g, gagCpolIna; h, gagCpol; i, RT; k, protInaRT; l, p2polIna.

<10 µg were adjusted to 10 µg by using noncoding vector pCMVkm2 as carrier DNA to avoid possible negative effects on immune potency that have been observed at low DNA doses (G. R. Otten, unpublished results). Table 1 contains a summary of the mouse studies performed and the immunization regimens used.

Measurements of antibody responses to p24^{Gag}. Plates (96 wells; Corning) were coated with 100 µl of recombinant HIV-1_{SF2} p24^{Gag} antigen (Chiron) at a concentration of 2 µg/ml in 50 mM borate buffer, pH 9. Sera were diluted 1:25 and then serially diluted threefold in dilution buffer containing 1% casein as a blocking reagent. Pooled anti-p24^{Gag} antibody-positive mouse sera served as both positive controls and assay standards. All sera were incubated for 1 h at 37°C, washed, and incubated with a 1:20,000 dilution of goat anti-mouse IgG plus IgM peroxidase conjugate (Pierce) for 1 h at 37°C. After washing of the plates, the tetramethylbenzidine substrate (Pierce) was added to each well and the reaction was stopped after 30 min by addition of 1 M H₃PO₄. The plates were read on an ELISA reader (312e; Bio-Tek Instruments, Inc., Winooski, Vt.) at 450 nm with a reference wavelength of 600 nm. The calculated titers are the reciprocal of the dilution of serum at a cutoff optical density of 0.4.

Challenge of immunized mice with recombinant vaccinia viruses (rVVs) expressing Gag or Pol. Challenge of gag DNA-primed mice with rVV expressing HIV-1_{SF2} GagPol (with frameshift) (B. Doe and C. Walker, Letter, AIDS 10: 793-794, 1996) can enhance humoral and cellular immune responses to Gag compared to those observed after DNA immunization alone (Otten, unpublished). Thus, the rVVGagpol challenge model can provide a useful means by which to obtain quantitative measurements of antigen-specific CD8⁺ T-cell function (Otten, unpublished). Mice were challenged 28 days postimmunization with an intraperitoneal injection of 10⁷ PFU of rVV. Spleens were removed 5 days later, and spleen cells were isolated for further evaluation in an intracellular cytokine-staining (ICS) assay (described below). An rVV expressing HIV-1_{SF2} Pol was constructed to allow application of this challenge model for the measurement of Pol-specific T-cell responses. Because of the frameshift in *gagpol*, the expression of Pol was insufficient if rVVGagpol was used. The complete codon-optimized *pol* sequence, with the exception of *integrase*, was used. Protease and RT were left functional. The gene was cloned into the shuttle vector pSC11 (11) via *Xma*C1 and *Hind*III sites, and rVV expressing Pol was generated as described for rVVGagpol.

ICS for Gag- and Pol-specific IFN-γ-producing CD8⁺ lymphocytes. Stimulation and staining of isolated spleen cells were done as described previously (56). Briefly, spleens were harvested 2 weeks post second DNA immunization or 5 days post rVV challenge and single-cell suspensions were prepared. Nucleated spleen cells (10⁶) were cultured in duplicate at 37°C in the presence or absence of 10 µg of p7g peptide per ml (Doe and Walker, letter) for Gag or by using the RT39-47_{SF2} peptide TEMEGKEKI (35) for the stimulation of Pol-specific CD8⁺ cells. Unstimulated cells plus spleen cells from naive mice were used as background and negative controls. The background values were generally very low, between 0.01 and 0.1% of IFN-γ-secreting CD8⁺ cells. After 5 h, cells were washed, incubated with anti-CD16/32 (Pharmingen, San Diego, Calif.) to block Fcγ receptors, fixed in 1% (wt/vol) paraformaldehyde, and stored overnight at 4°C. On the following day, cells were stained with fluorescein isothiocyanate-conjugated CD8 MAb (Pharmingen), washed, treated with 0.5% (wt/vol) saponin (Sigma), and then incubated with phycoerythrin-conjugated mouse IFN-γ MAb (Pharmingen) in the presence of 0.1% (wt/vol) saponin. Cells were then washed

and analyzed on a FACScalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, Calif.).

Statistical analysis of Gag- and Pol-specific IFN-γ-producing CD8⁺ T-cell responses. For analysis of the relative CD8⁺ T-cell responses in the mouse immunogenicity studies, a regression analysis was performed. Each regression analysis began with a single regression model incorporating indicator variables to allow for individual intercepts and slopes specifically for each treatment. The model is $Y_i = \beta_0 + \beta_0\delta_i + \beta_1x + \beta_1\delta_1x + \epsilon$, where $i = 1 \dots \text{no. of treatments}$. Here Y_i is the log₁₀ background-corrected percentage of cells showing a positive CD8 IFN-γ response for peptide treatment group i and HIV DNA vaccine dose level x . The intercept for each treatment is the overall intercept, β_0 , plus an additional term, $\beta_0\delta_i$, for treatment i . The slope for each treatment is β_1x plus an additional term, $\beta_1\delta_1x$. The δ_i values are indicator variables that equal 1 for treatment i and are 0 otherwise. The model was iteratively reduced by removing first nonsignificant slope terms, those with $P > 0.05$, and then nonsignificant intercept terms, those with $P > 0.05$, in the reduced-slope model. The result was a final regression model with only the significant slope and intercept terms, those with $P < 0.05$. This model-building process was repeated for each of seven experiments, corresponding to Fig. 4, 5A and B, and 7A to D. Scatter plots for each figure including the significant regression model equations for each treatment were plotted by using SPLUS 2000.

RESULTS

Construction of novel gag- and pol-derived expression cassettes. Previously, we reported on the construction and characterization of a sequence-modified Gag plasmid that was found in several studies to be a potent inducer of Gag-specific immune responses (38, 56). In the present work, we sought to broaden the spectrum of viral epitopes represented in our DNA vaccine approach (without introducing a reduction of Gag-specific immune responses) through the addition of Pol coding sequences. For this purpose, we designed and evaluated several novel gag and pol expression cassettes. A summary of the sequence-modified gene cassettes evaluated here is shown in Fig. 1. The constructs GagMod (gag), GP1, and GP2 were described and characterized previously but are included for comparison (56). The gene cassette gagFSpol was based on GP2 with an extension of Pol including the p66^{RT} coding region but without the integrase coding sequences. The integrase was excluded from all of the constructs described here to avoid possible integration of vaccine sequences into the host genome. To improve Pol expression, the frameshift region between the gag and pol genes was mutated by single-base insertion to create gagpol with both the Gag and Pol coding sequences in the same open reading frame. Creation of this

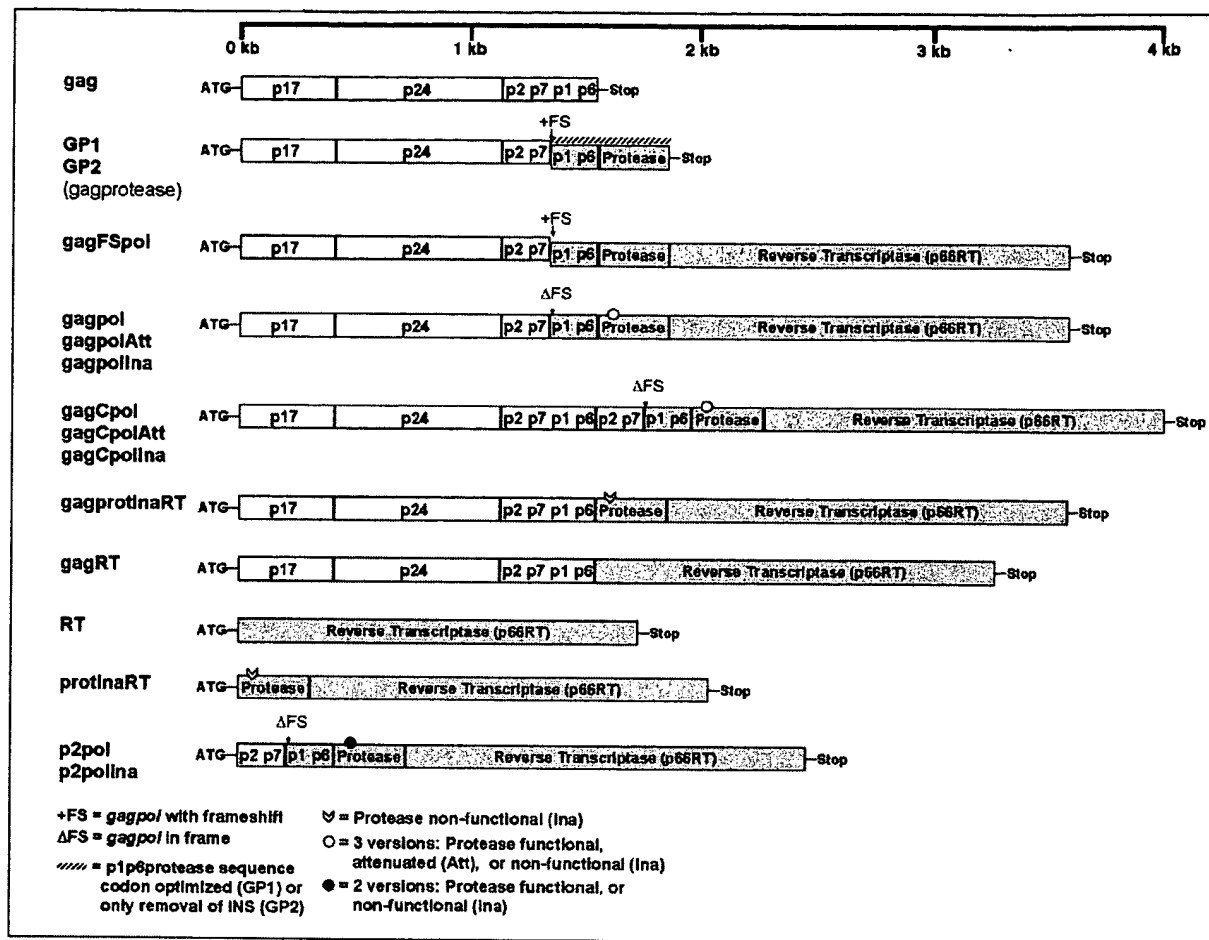


FIG. 1. Overview of HIV-1 *gag* and *pol* expression cassettes. All sequences are based on the HIV-1_{SF2} isolate (GenBank accession no. K02007) and were optimized for human codon usage. The coding sequence for RT was mutated for all affected constructs to yield a nonfunctional protein. The various versions of constructs with mutations to eliminate the frameshift (FS) and protease (Prot) activity are shown.

construct resulted in the loss of p1p6^{Gag} because of the mutation introduced to remove the frameshift. Because the p6 portion of Gag was shown to be important for the efficient release of Gag VLP (19), the cassette gagCpol was designed to include a repeat of p2p7p1p6 to restore p1p6Gag expression. Moreover, the p2p7^{Gag} repeat was introduced to improve the secretion and autoprocessing of gagCpol by the protease (1, 57). Also, to enhance possible processing requirements for efficient expression, a *pol* cassette was designed to include p2p7gag (p2pol and p2pol). Because of concerns regarding potential cytotoxic properties of the functional viral protease (32) that could affect both antigen expression and immunogenicity, the *protease* gene was either attenuated (Att) or rendered inactive (Ina) in the designated constructs (Fig. 1). Fusion cassettes expressing Gag plus RT (gagRT) and Gag plus protease plus RT (gagprotInaRT) were also constructed and compared to gagCpol.

In vitro characterization of expression cassettes. To evaluate the expression patterns of the various Gag- and Pol-containing constructs, 293 cells were transiently transfected and supernatants and cell lysates were analyzed by p24^{Gag} antigen

capture ELISA and immunoblotting. Because the p24^{Gag} antigen capture ELISA preferentially recognizes processed forms of Gag (48, 56), comparative expression analyses were problematic to perform for all constructs. However, comparison of very similar constructs allowed us to test for differences in Gag expression.

Figure 2 illustrates the relative Gag expression levels. The cassette gagFSpol was designed to extend the Pol region and at the same time maintain the natural processing and frameshift translation of the expressed GagPol precursor polypeptide. In cell lysates, the expression level of Gag from this construct was about the same as that of Gag expressed by GP2 (Fig. 2B) but about fourfold less p24^{Gag} was detected in the culture supernatant compared to that of GP2 (Fig. 2A). In the gagpol and gagCpol constructs, the frameshift sequences were altered so that Gag and Pol could be expressed by the same reading frame in order to increase the expression of Pol without affecting Gag expression. In alternative versions of these constructs, the *protease* gene was either mutated to produce attenuated (gagpolAtt, gagCpolAtt) or inactivated (gagpolIna, gagCpolIna) protease. As shown in Fig. 2C, no differences in

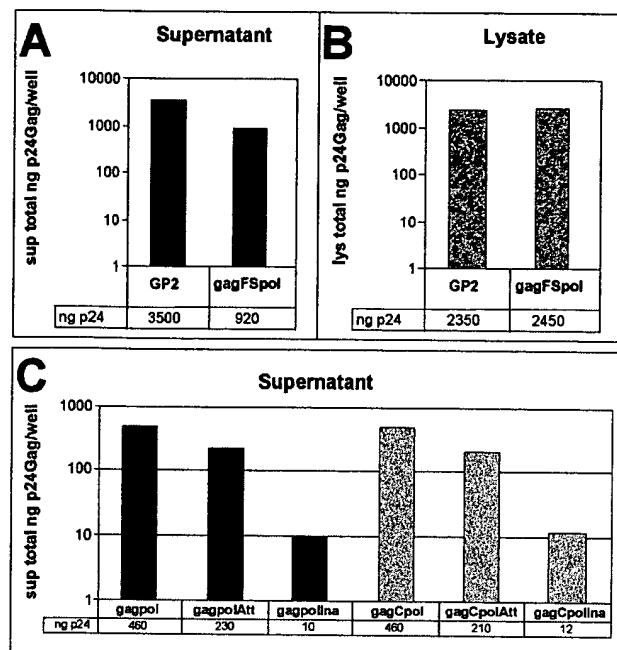


FIG. 2. Quantitative comparison of HIV-1 Gag expression by p24^{Gag} antigen capture ELISA of supernatants (sup) and lysates (lys) of 293 cells 48 h posttransfection with *gagpol* constructs. Supernatants (A) and lysates (B) of GP2 versus gagFSpol are shown. Both cassettes express functional protease with an intact frameshift. In the experiment whose results are shown in panel C, supernatants were analyzed from different versions of in-frame gagpol versus gagCpol with functional, nonfunctional, or attenuated protease. The gagCpol cassettes included an additional *p2p7p1p6^{gag}* sequence.

p24^{Gag} levels were observed in culture supernatants when similar versions of gagpol and gagCpol were compared. The same results were also obtained with the cell lysates (data not shown). Thus, the additional insertion of the *p2p7p1p6* fragment appeared to have no influence on p24^{Gag} expression levels as measured here.

Western blot analysis was performed with all of the expression cassettes described in Fig. 1 by using Gag-specific, HIV-positive human antisera (Fig. 3A and B). Clear differences were observed between plasmids expressing processed and unprocessed forms of the Gag and GagPol polyproteins. The highest level of Gag-specific reactivity appeared to be found in supernatants (Fig. 3A) and lysates (Fig. 3B) of cultures of cells transfected with gag, followed by GP2 and gagFSpol (data not shown). GP2 and gagFSpol process the Gag polyprotein by using a protease that is underexpressed with the natural frameshift intact, and the bands observed included unprocessed p55^{Gag} and processed forms of Gag. As would be expected in the absence of protease, very little or no processed p24^{Gag} was seen in lysates of cells expressing Gag alone; nevertheless, the small amount of processing observed in the supernatants of these cells was likely due to the presence of nonspecific cellular protease activity. In transfections with two of the constructs expressing Gag and Pol in the same reading frame, gagCpol and gagCpolAtt, the band corresponding to p55^{Gag} was not detectable in the cell supernatants or lysates and reduced

amounts of p24^{Gag} were seen in supernatants and lysates (Fig. 3A and B). For gagCpolIna with the nonfunctional protease, no Gag-specific bands were detected in cell supernatants (Fig. 3A) and a high-molecular-mass band corresponding to the unprocessed GagCPol polyprotein (149 kDa) was observed to migrate as expected in the cell lysate (Fig. 3B). Additional bands expressed from gagCpolIna included small amounts of p55^{Gag} and p41^{Gag}, but no p24^{Gag} could be detected. Accordingly, when cells transfected with gagCpolIna, gagCpol, and gag were examined by electron microscopy, very few VLP were detected for gagCpolIna and no particles were detected for gagCpol, indicating impaired secretion of VLP compared to that achieved with gag (data not shown). The cassettes gagRT (121 kDa) and gagprotInaRT (131 kDa) showed levels of Gag comparable to those observed for gagCpolIna (data not shown).

The expression of Pol in cell lysates from transfected 293 cells was also analyzed by Western blotting with RT-specific antisera (Fig. 3C). In general, both the single-gene cassettes in the absence of Gag (RT, proteaseRT, and p2polIna) and the *gagpol* fusion cassettes (gagRT, gagprotInaRT, gagCpolIna, and gagpolIna) appeared to be expressed well as long as the *protease* gene was absent or nonfunctional. The RT (66 kDa) and protInaRT (75 kDa) cassettes appeared to be expressed at the highest levels, followed by the p2polIna (93 kDa), gagRT (121 kDa), and gagprotInaRT (131 kDa) cassettes, followed by the gagCpolIna (149 kDa) and gagpolIna (132 kDa) cassettes. The latter two constructs exhibited high-molecular-weight bands of the expected relative mobilities (and slightly faster, respectively) of similar intensities indicative of comparable levels of expression. In constructs expressing the functional and attenuated HIV protease, p2pol, gagCpol, and gagCpolAtt, reduced expression of RT-specific bands was observed compared to the levels expressed by the p2polIna and gagCpolIna constructs. In summary, the addition of *gag* sequences to *pol* appeared to have very little influence on Pol-specific expression levels and vice versa but the addition of a functional *protease* gene resulted in reduced expression of Gag- and RT-specific bands.

Design of mouse immunogenicity studies. The relative immunogenicities of the DNA plasmids encoding the various gene cassettes were evaluated in mice that were intramuscularly immunized with doses of plasmid DNA ranging from 0.002 to 20 μ g (Table 1 contains a summary of the studies performed). This afforded a determination of the dose dependency for each plasmid. In each experiment, groups of 4 to 10 mice were immunized per dose of a given plasmid. One set of mice was immunized twice, at weeks 0 and 4, with spleen removal and analysis at week 6, and another set was immunized once with DNA and then challenged after 4 weeks with rVV expressing GagPol or Pol. Spleens were removed 5 days later, and cells were harvested for ICS to measure Gag- and Pol-specific IFN- γ -producing CD8⁺ lymphocytes. Because boosting with rVV enhanced specific immune responses to these antigens, T-cell responses could be evaluated after a single DNA prime even at the lowest DNA dose.

CD8⁺ T-cell responses to Gag. Gag-specific CD8⁺ T-cell responses were analyzed by intracellular IFN- γ staining of CD8⁺ spleen cells that had been stimulated with Gag peptide p7g, an H-2K^d-restricted epitope (Doe and Walker, letter). In

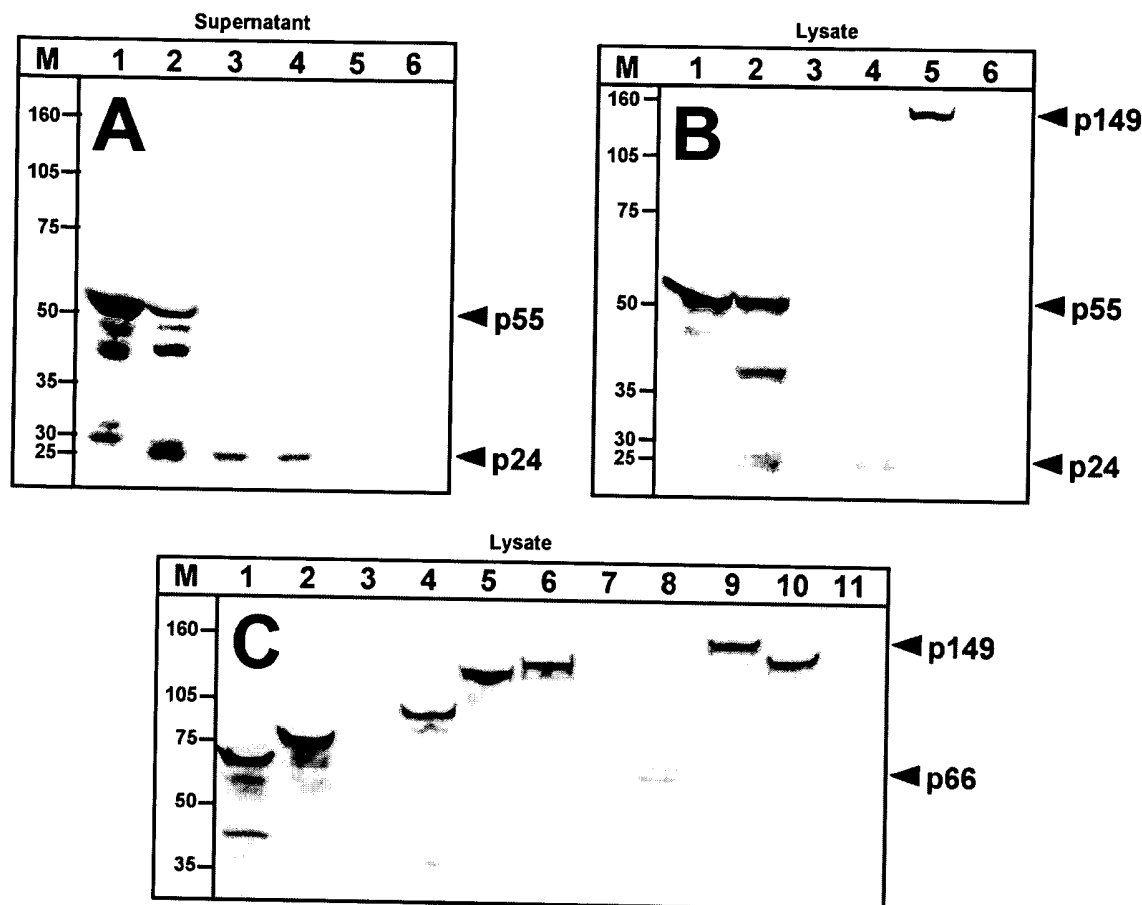


FIG. 3. Immunoblots of synthetic HIV-1 *gag* and *pol* expression cassettes. 293 cells were transfected, and supernatants and lysates were collected 48 h posttransfection, subjected to 4 to 12% Bis-Tris sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and blotted onto nitrocellulose membranes. Immunostaining was performed with either human HIV-1 patient serum (A and B) or pooled anti-p66^{RT} mouse serum (C). For detection of Gag expression, supernatants (A) and lysates (B) were used. Lanes: 1, *gag*; 2, GP2; 3, *gagCpol*; 4, *gagCpolAtt*; 5, *gagCpolIna*; 6, mock transfection. For detection of Pol products, only cell lysates were analyzed (C). Lanes: 1, RT; 2, *protInaRT*; 3, *p2pol*; 4, *p2pollna*; 5, *gagRT*; 6, *gagprotInaRT*; 7, *gagCpol*; 8, *gagCpolAtt*; 9, *gagCpolIna*; 10, *gagpollna*; 11, mock transfection. The values on the left are molecular sizes (M) in kilodaltons.

the first study (Fig. 4), addition of functional protease to Gag with a frameshift in constructs GP1 and GP2 was tested. The CD8⁺ T-cell responses after two DNA immunizations were indistinguishable for all three plasmids. Thus, from these results, protease-mediated cleavage of Gag apparently did not affect the processing and presentation of Gag in vivo. At the lowest plasmid dose (0.02 μ g), Gag-specific CD8⁺ T cells were only 30 to 50% below maximum. Therefore, for the next studies, the lowest DNA dose was reduced further to 0.002 μ g. Furthermore, new constructs were included and compared to *gag*. The potency of all of the plasmids tested with regard to the induction of Gag-specific CD8⁺ T cells was indistinguishable after a single DNA immunization followed by an rVVgagpol challenge or after two DNA immunizations (Fig. 5A and B).

Addition of *pol* sequences to *gag* in the DNA vaccine constructs evaluated here did not affect the induction of Gag-specific immune responses. Moreover, despite apparent differences between *gagCpol* and *gagCpollna* in Gag expression as measured in vitro (Fig. 3), the induction of Gag-specific CD8⁺ T-cell responses was not affected by functional protease.

Antibody responses to Gag. The measurement of Gag-specific antibody responses revealed a different pattern of responses for the various constructs compared to that observed for the cellular responses. In the first experiment, a comparison was drawn between *gag* and GP1 and GP2 (Fig. 6A) to look for possible effects of the functional protease on the immunogenicity of p55^{Gag} when protease is expressed with the natural frameshift. The p55^{Gag} antibody responses at 2 weeks post second DNA immunization demonstrated the overall weakest responses with GP1 and better responses with GP2. The *gag* DNA appeared to be more immunogenic, especially at the lower DNA doses, but GP2 was more comparable to *gag* at the highest DNA dose (20 μ g). For the next experiment (Fig. 6B), antibody responses were analyzed 5 days after a vaccinia virus challenge. Additional cassettes expressing Gag and Pol in frame (*gagprotInaRT*, *gagCpol*, and *gagCpolIna*) were evaluated. In comparison to the previously described analysis (Fig. 6A), the differences between constructs were much more apparent. Two patterns of antibody induction emerged. The *gag* and *gagFSPol* cassettes induced strong humoral immune re-

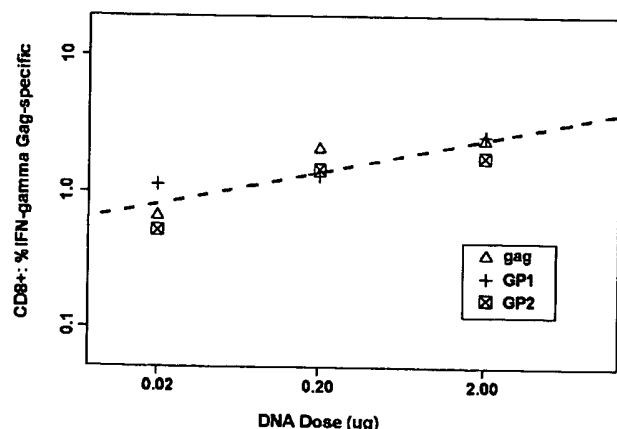


FIG. 4. Quantitative analysis of Gag-specific, IFN- γ -secreting CD8 $^{+}$ T cells. CB6F1 mice were immunized twice at weeks 0 and 4 with titrated doses of codon-optimized HIV-1_{SF2} gag, GP1, and GP2 plasmid DNAs. Spleens were removed 2 weeks after the second immunization, and the pooled spleen cells were stimulated in duplicate for 5 h with the p7g peptide. On the following day, cells were stained for CD8 $^{+}$ and intracellular IFN- γ and analyzed by flow cytometry. Data were analyzed by a regression model (see Materials and Methods for details).

sponses post vaccinia virus challenge, while the gagprotInaRT, gagCpol, and gagCpolIna cassettes were much less potent for the induction of antibody titers. The observed antibody responses appear to correlate with the relative amounts of secreted Gag proteins observed in the *in vitro* analysis (Fig. 3). The constructs that secreted the highest levels of Gag (gag and gagFSpol) primed for the most potent antibody responses, while those that expressed high-molecular-weight polyproteins in the cell lysates (gagprotInaRT and gagCpolIna) or overprocessed Gag (gagCpol) induced the poorest antibody responses.

Cellular immune responses to Pol. For detection of cellular immune responses to Pol, studies were done with C3H/HeN mice. Spleen cells were stimulated with the H-2K k -restricted nonamer TEMEKGEKI (35) and analyzed by flow cytometry

for IFN- γ synthesis. Figure 7A and C compare the RT and protInaRT DNA vaccines with those encoding gag plus pol sequences. In general, the magnitude of the Pol responses was lower than that of the Gag responses. No significant differences were observed between the different antigens, with the exception of gagFSpol, which was not as potent as expected as a result of the low-level expression of the encoded Pol products. Figure 7B and D show that the p2pol cassette, in which the p2p7gag and p1p6pol sequences precede protInaRT, induced Pol-specific CD8 $^{+}$ T cells, even at low doses. Thus, in-frame insertions of p2p7p1p6 and protease upstream of RT did not seem to reduce RT-specific immunogenicity. To study this further, the complete gag coding region was inserted upstream of pol. As shown in Fig. 7, in-frame insertion of gag did not suppress the induction of RT-specific CD8 $^{+}$ T cells; however, if the wild-type frameshift was present (gagFSpol), the vaccine was less potent at inducing this Pol-specific response after a vaccinia virus boost for all doses (Fig. 7B) and no response was detectable after two DNA immunizations, even at the highest dose (Fig. 7D). As for immune responses to Gag, the differences in Pol expression in gagCpol constructs with functional and nonfunctional protease, as seen *in vitro*, did not result in differences in the observed immune potencies of these constructs. The cellular immune responses to Pol, as measured here, were not affected either by the activity of protease or by the addition of gag sequences upstream of pol.

DISCUSSION

For the design and development of an effective HIV-1 vaccine, the induction of T-cell responses with a large repertoire of specificities is essential. Inclusion of HIV-1 Pol in a vaccine would be expected to increase this repertoire significantly (54). Pol is well-conserved, broad CTL responses are found in the majority of infected patients, and these responses have been shown to be inversely correlated to the viral load (7, 21). Since the virus-encoded pol gene is expressed at very low levels compared to gag as a result of the translational frameshifting mechanism by which it is expressed, increasing pol expression

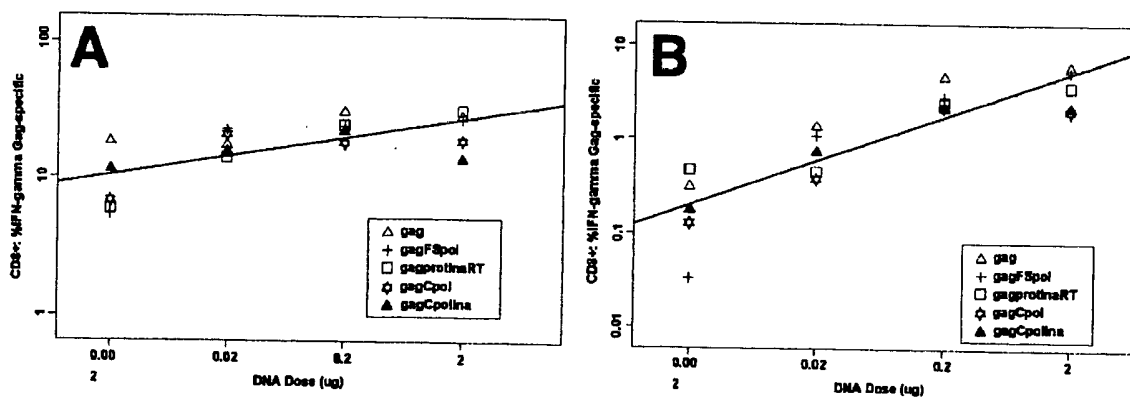


FIG. 5. HIV-1_{SF2} Gag-specific CD8 $^{+}$ responses of CB6F1 mice immunized with titrated DNA doses of gag or gag-plus-pol cassettes. Groups of mice were either immunized once and challenged with rVVgagpol 4 weeks later (A) or received two immunizations with DNA at weeks 0 and 4 (B). Spleens were harvested 5 days post vaccinia virus challenge or 2 weeks post second immunization, respectively. Pooled splenocytes were stimulated with the Gag-specific peptide p7g for 5 h. Cells were stained for CD8 $^{+}$ and intracellular IFN- γ on the next day and analyzed by flow cytometry. Data were analyzed by a regression model (see Materials and Methods for details).

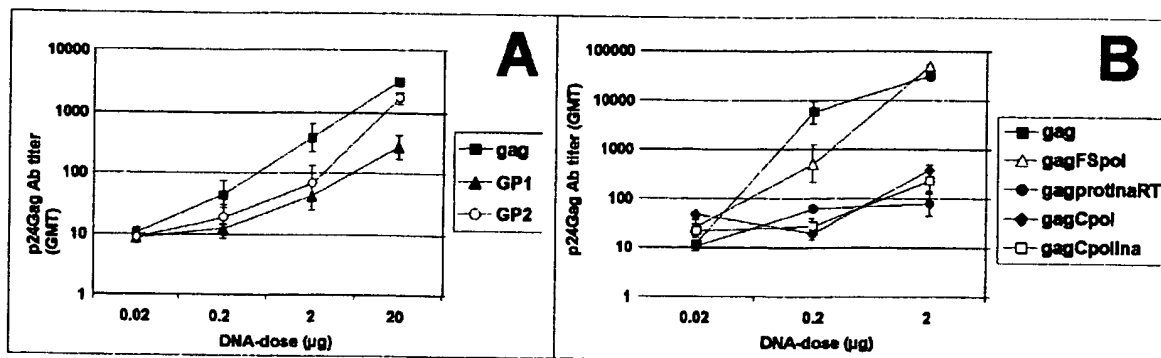


FIG. 6. Antibody (Ab) titers specific for HIV-1_{SF2} p24^{Gag} in mice 2 weeks after two immunizations (weeks 0 and 4) with DNA (A) or 5 days postchallenge with rVVgagpol after a single DNA immunization at week 0 (B). Collected serum samples were analyzed by p24^{Gag} ELISA as described in Materials and Methods. (A) The plasmid expressing only p55^{Gag} (gag) was compared to GP1 and GP2. (B) Expression cassettes gag and gagFSpol were compared to nonframeshifted versions of gagpol. The values shown are the geometric mean antibody titers and the standard deviations of the midpoint antibody titers for each group.

by removal of the natural frameshift and removal of inhibitory sequences could result in the induction of a higher frequency of Pol-specific effector and memory CTL by *pol*-based DNA vaccines. In addition, because an effective HIV-1 vaccine would very likely be composed of at least *gag* and *pol* plus *env*, cost and practicability should also be considered. A multigenic

DNA vaccine containing *gag* and *pol* on one plasmid would therefore be an advantage. Gene cassettes encoding *gagpol* have been used previously in vaccines with modest immunological outcomes with respect to the induction of Pol-specific T-cell responses in human and nonhuman primate studies (9, 15, 16). This could be explained by the use of the *gagpol* gene

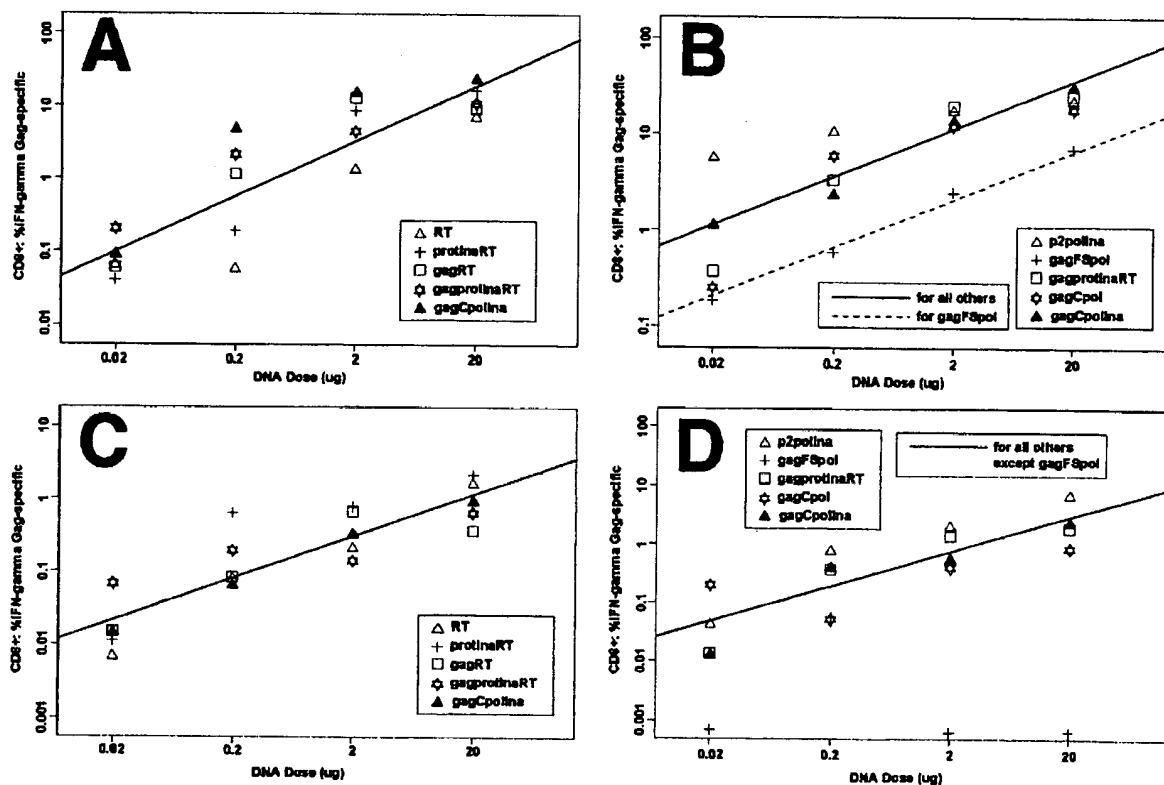


FIG. 7. Frequencies of HIV-1_{SF2} RT-specific CD8⁺ T-cell responses of C3H/HeN mice immunized with titrated DNA doses of RT, protinaRT, p2polina, or *gag*-plus-*pol* cassettes. Results for mice immunized once and challenged with rVVpol 4 weeks later are shown in panels A and B. Another set of animals received two DNA immunizations at weeks 0 and 4 (C and D). Spleen harvesting and staining for flow cytometry were performed as described in the legend to Fig. 5. Data were analyzed by a regression model (see Materials and Methods for details).

with an intact frameshift and/or native codon usage, which would be expected to provide lower levels of *pol* expression. Casimiro et al. reported recently for the first time strong Pol-specific cellular immune responses in nonhuman primates after immunization with synthetic *pol* DNA vaccines (10).

In this work, we analyzed immune responses to HIV-1 *gag* and a variety of *pol* sequences in separate and combined expression cassettes. Particular attention was given to the possible negative effect of *pol* on *gag* expression and immunogenicity. Immune responses to the well-characterized plasmid pCMVkm2.GagMod.SF2 (*gag*) (56) served as a benchmark for these studies. Results obtained with the sequence-modified *pol* gene indicated that the expression and immunogenicity of Gag using gagFSpol with an intact frameshift was not affected by the *pol* sequence (Fig. 5 and 6B). Also, after removal of the frameshift region from the *gagpol* cassettes, Pol expression was improved dramatically. While Pol expression could not be detected in Western blots of lysates and culture supernatants from cells transfected with gagFSpol (data not shown), plasmids encoding an in-frame *gagpol* cassette with nonfunctional protease showed high-level expression (Fig. 3C). This was also confirmed in mice immunized with gagFSpol versus gagCpol in-frame cassettes. Pol-specific CD8⁺ T-cell responses could only be detected in gagFSpol-immunized animals after an rVVpol boost, whereas gagCpol induced strong responses after two DNA doses (Fig. 7B and D). Interestingly, previously described cytotoxic effects of HIV-1 protease that were shown to affect the expression of additional genes in vivo (51) did not diminish CD8⁺ T-cell responses. The gagCpol (functional protease) and gagCpolIna (nonfunctional protease) DNA vaccines were indistinguishable in their abilities to induce cellular immune responses to Gag or Pol (Fig. 5 and 7B and D). However, reduced expression of the Gag and Pol proteins was observed in Western blots of transfected cells when the protease was functional (Fig. 3). Whether this effect was directly related to negative effects of protease or altered expression kinetics remains to be determined.

HIV-1 Gag is a major target with respect to the induction of CTL responses in HIV-1-infected patients, and p24^{Gag} and p17^{Gag} appear to have the highest epitope density, besides Nef, of all HIV-1 antigens (55). Recently, an important contribution of p15^{Gag} to the overall CTL response in HIV-1-infected subjects also was reported (55). This result should be considered in a Gag-based vaccine design. Thus, to retain important epitopes for Gag, the gagCpol cassette, containing the complete *gag* coding sequences in addition to *pol* in frame, was designed. After removal of the frameshift by a single-base insertion, p1p6^{Gag} protein expression was lost, resulting in a truncated Gag protein that was shortened by p1p6^{Gag} at the frameshift site. The extension of *gagpol* to include p2p7p1p6^{Gag} in the gagCpol construct had no negative influence on expression (Fig. 2C), and this cassette design was therefore selected for use in immunogenicity studies instead of the original *gagpol* construct.

Immune responses generated against Gag or Pol by using various Gag- and Pol-expressing DNA vaccines were evaluated by repeated experiments with either two DNA immunizations or one immunization followed by an rVV boost. Responses were scored by flow cytometric measurements of antigen-specific IFN- γ -secreting CD8⁺ cells with an ICS assay. Responses

to Gag were detectable after two immunizations with amounts of DNA as small as 2 ng. No significant differences in Gag-specific CD8⁺ T-cell responses were found for any of the sequence-modified expression cassettes tested here. Cellular immune responses to Pol were analyzed by using C3H mice (*H-2^k*), and spleen cells were stimulated by using the 9-mer CTL peptide described by Hosmalin et al. (25). Positive responses could be detected in the 20- to 200-ng DNA dose range, compared to 2 ng for Gag. This could be explained by the reduced recognition and assay sensitivity of this peptide as recently described (10). However, solid stimulation was demonstrated with this peptide epitope; up to 32% of RT-specific CD8⁺ cells responded after one 20- μ g DNA prime and an rVV boost (Fig. 7A and B). As expected from the expression results, the gagFSpol DNA vaccine (i.e., Pol expressed with a frameshift) induced significantly lower levels of Pol-specific immune responses if DNA-primed mice were boosted with rVV expressing Pol (Fig. 7B) and no detectable Pol-specific responses after two DNA immunizations (Fig. 7D). As for Gag responses, no significant differences were found among the in-frame sequence-modified constructs with regard to the induction of Pol-specific CD8⁺ T-cell responses. Thus, it appears that efficient secretion of Gag antigens as VLP secretion, which is impaired in *gagpol* fusion constructs (28, 40), was not essential for the induction of potent Gag-specific CD8⁺ responses. Previous results obtained by another group using synthetic *pol* and *gagpol* genes also demonstrated improved expression of Pol when it was fused in frame with Gag (26). However, cellular immune responses to Gag and Pol were demonstrated for single and fusion gene cassettes when mice were immunized four times with 100 μ g of DNA. In our experiments, we titrated the DNA doses down to 2 ng for Gag responses and 20 ng for Pol responses, which allowed us to more fully evaluate the relative potency of each construct. Moreover, in the present study, several additional versions of *pol* and *gagpol*, including those with an attenuated, functional and nonfunctional protease gene, were analyzed.

Altogether, the data presented in this study suggest that the highly efficient expression and immunogenicity of Gag are not impaired by Pol, and vice versa, if Gag and Pol are expressed as a multigenic fusion protein (gagCpol) in a DNA vaccine. Moreover, the expression and immunogenicity of the Pol antigen can be enhanced through removal of the frameshift and sequence modifications to remove inhibitory sequences and optimize codon usage. The improved *gag-plus-pol* DNA vaccine described here, when administered by using recently described enhanced DNA vaccine delivery technologies (38, 52), should prove to be a potent vaccine for the induction of T-cell immune responses. Furthermore, vaccine approaches that combine the gagCpol DNA vaccine for the induction of cellular immune responses with improved Env antigens for the induction of neutralizing antibodies (3, 13, 50) hold great promise for the next generation of HIV vaccines.

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EXHIBIT C

Vaccine. 1997 Jun;15(8):884-7.

Related Articles, Links

Anti-HIV env immunities elicited by nucleic acid vaccines.**Shiver JW, Davies ME, Yasutomi Y, Perry HC, Freed DC, Letvin NL, Liu MA.**

Department of Virus and Cell Biology, Merck Research Laboratories, West Point, PA 19486, USA.

Plasmid DNA vaccines encoding HIV-1 env were used to immunize mice and nonhuman primates. Plasmids were prepared that produced either secreted gp120 or full-length gp160. Mice immunized with gp120 DNA developed strong antigen-specific antibody responses, CD8+ cytotoxic T lymphocytes (CTL) (following in vitro restimulation with gp120-derived peptide), and showed in vitro proliferation and Th1-like cytokine secretion [gamma-interferon, interleukin (IL)-2 with little or no IL-4] by lymphocytes obtained from all lymphatic compartments tested (spleen, blood, and inguinal, iliac, and mesenteric lymph nodes). This indicated that systemic anti-gp120 cell-mediated immunity was induced by this DNA vaccine. Although similar antibody responses were observed in mice immunized by either intramuscular or intradermal routes, T cell responses were significantly stronger in mice injected intramuscularly. Rhesus monkeys immunized with both gp120 and gp160 DNAs exhibited significant CD8+ CTL responses, following in vitro restimulation of peripheral blood lymphocytes with antigen. These experiments demonstrate that DNA immunization elicits potent immune responses against HIV env in both a rodent and a nonhuman primate species.

PMID: 9234539 [PubMed - indexed for MEDLINE]

EXHIBIT D

J Immunol Methods. 1998 Nov 1;220(1-2):93-103.

Related Articles, Links

Intranasal immunization with a plant virus expressing a peptide from HIV-1 gp41 stimulates better mucosal and systemic HIV-1-specific IgA and IgG than oral immunization.

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Control of pandemic human immunodeficiency virus type 1 (HIV-1) infection ideally requires specific mucosal immunity to protect the genital regions through which transmission more often occurs. Thus a vaccine that stimulates a disseminated mucosal and systemic protective immune response would be extremely useful. Here we have investigated the ability of a chimeric plant virus, cowpea mosaic virus (CPMV), expressing a 22 amino acid peptide (residues 731-752) of the transmembrane gp41 protein of HIV-1 IIIB (CPMV-HIV/1), to stimulate HIV-1-specific and CPMV-specific mucosal and serum antibody following intranasal or oral immunization together with the widely used mucosal adjuvant, cholera toxin. CPMV-HIV/1 has been shown previously to stimulate HIV-1-specific serum antibody in mice by parenteral immunization. All mice immunized intranasally with two doses of 10 microg of CPMV-HIV/1 produced both HIV-1-specific IgA in faeces as well as higher levels of specific, predominantly IgG2a, serum antibody. Thus there was a predominantly T helper 1 cell response. All mice also responded strongly to CPMV epitopes. Oral immunization of the chimeric cowpea mosaic virus was less effective, even at doses of 500 microg or greater, and stimulated HIV-1-specific serum antibody in only a minority of mice, and no faecal HIV-1 specific IgA.

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